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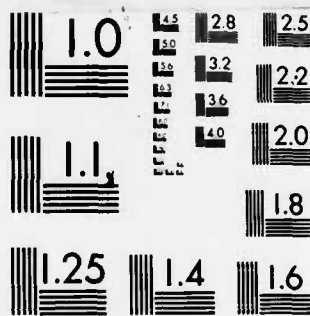
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HUMAN PLATELET SENESENCE STUDY

Final Summary Report

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March 1980

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ABSTRACT (continued)

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ABSTRACT

A technique was developed for the identification of platelet membrane antigens. This involves agar electrophoresis in one direction, followed by rotation of the slide 90° and electrophoresis into agar containing anti-platelet membrane antibody. Nineteen different platelet membrane antigens (immunoprecipitates) can be detected. These include: albumin, fibrinogen and an antigen absent in platelets from patients with Glanzmann's thrombasthenia. The relative cell surface antigens can be detected by absorption studies with intact platelets. The presence of carbohydrate can be detected with suitable lectins, such as concanavalin A used in an intermediate spacer gel.

Colchicine, an antimicrotubule agent stimulates dephosphorylation of phosphorylated platelet cell sap. This effect is inhibited by D₂O, a microtubule stabilizing agent. Similar results are obtained with cyclic AMP. It is suggested that colchicine operates via activating an adenylate cyclase, which is inhibited by microtubules.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

For the past year our laboratory has been engaged in two areas of platelet (and megathrombocyte) research with regard to: heterogeneity and senescence as related to microtubules, protein phosphorylation, platelet aggregation and secretion, and platelet membrane topography.

1. Crossed Immunoelectrophoresis of Platelet Membranes.
2. Requirement of Microtubules for Platelet Aggregation and Secretion; Association of Phosphodiesterase Activity with Platelet Tubulin.

SUMMARY OF PROJECTS

1. Crossed Immunoelectrophoresis of Platelet Membranes.

Early studies on platelet membranes, employing SDS-PAGE, revealed the presence of three major glycoproteins, GPI, GPII, and GPIII, with apparent molecular weights of 155,000, 135,000 and 103,000 (1-3). In 1974 Nurden and Caen, employing SDS-PAGE and PAS carbohydrate staining, demonstrated the absence of GPII and the diminished presence of GPIII in 3 patients with Glanzmann's thrombasthenia (3). This was confirmed by Phillips et al (4) who noted a diminished content of GPII rather than its absence. In 1975 Nurden and Caen also demonstrated absent-to-trace amounts of GPI in patients with Bernard-Soulier disease (5) and suggested that GPI was the receptor for Von Willebrand factor. Nurden and Caen also demonstrated that GPI was rich in sialic acid (5) and probably responsible for the platelets' surface charge. More refined techniques for demonstrating platelet membrane glycopeptides have subsequently demonstrated the presence of at least 6 to 7 surface glycopeptides (6): GPIs, GPIb, GPIc (non-PAS positive), GPIIa, GPIIb, GPIII and GPIV (87,000 m.w., also called GPIIb). The defect in Glanzmann's thrombasthenia has now been designated as diminished GPIIb and GPIIIa; the defect in Bernard-Soulier as diminished GPIs and GPIb. In 1978 Kunicki and Aster (7) presented evidence for the association of the platelet antigen PLAI with GPIIb or GPIIIa, since platelets from patients with Glanzmann's thrombasthenia do not release prelabelled Cr⁵¹ following addition of potent anti-PLAI antibody. These workers have recently presented evidence that PLAI resides on GPIIIa (8). Aster and co-workers later demonstrated that platelets from patients with Bernard-Soulier disease do not release prelabelled Cr⁵¹ following the addition of Ag-Ab complexes, suggesting the presence of the platelet Fc receptor on GPI (9). Similar conclusions were drawn by Nachman and co-workers (10) who noted competition between Fc binding of non-specific IgG and Von Willebrand factor. Jamieson and co-workers have purified soluble GPIs, which they have termed 'glycocalicin' (11), because of its origin in the platelet glycocalyx. They have suggested that GPIs is associated with the thrombin receptor since purified GPIs binds thrombin and inhibits thrombin-induced platelet aggregation; and platelets from patients with Bernard-Soulier disease aggregate poorly with low concentrations of thrombin (11-14).

Preliminary data from our laboratory suggest that specific platelet membrane proteins of lighter-older platelets are diminished-to-absent when examined by electrophoresis on 5% polyacrylamide gels in SDS (15). However, this technology requires large quantities of platelets from each subpopulation (approximately 4 to 5 units of original platelet-rich plasma) which must then be fractionated for platelet membranes by the techniques of Barber and Jamieson (16). The polyacrylamide gel SDS-electrophoresis technique is limited by its ability to measure denatured proteins at the 10-20 microgram level; as well as carbohydrate at a much higher level.

We have recently acquired a very sensitive technique for the measurement of platelet membrane constituents, which does not completely denature the membrane constituents, and which is 10-100 times more sensitive than SDS-PAGE. This is the technique of crossed-immunoelectrophoresis of plasma membranes, recently developed for the study of bacterial membranes by Dr. Salton (17), which gives a two-dimensional display of rockets or peaks of immunoprecipitates. The technology involves solubilization of platelet membranes in 1% Triton X-100 and electrophoresis in one direction on a 1% agarose slide in tris-barbital buffer, pH 8.6 at room temperature at 150 volts for 2 to 3 hours. Most of the agarose is then removed and a strip of the remaining electrophoresed constituents retained. Fresh agar, containing purified rabbit antibody against human platelet membranes, is then poured onto the slide and allowed to harden. The slide is rotated 90° and the antigens then electrophoresed into the agar containing the antibody at 55 volts for 16 hours. The agar is then pressed free

of excess fluid and washed in 0.1M NaCl to remove excess antibody; dried and stained with 0.25% Coomassie blue; and destained with 5% HAc-45% ethanol (Figure 1a-d). The advantages of this technique are: 1) the technique is essentially quantitative, the peak areas of individual immunoprecipitates being proportional to the antigen/antibody ratios; 2) high sensitivity of detection (e.g., on immunoelectrophoresis we can detect over 10 antigens); 3) ability to measure platelet membrane enzyme activity - by coupling certain enzymes to their oxidation-reduction reactions and nitro-blue tetrazolium dyes; other enzymes which can be measured by other techniques (catalase, lipase, alkaline phosphatase, acid phosphatase, chymotrypsin, trypsin, esterases (17)); 4) use of carbohydrate lectins such as concanavalin A or wheat germ agglutinin in the second dimension (instead of the antibody) to detect specific carbohydrate constituents.

We have developed this procedure with human platelet membrane antigens and can detect at least 19 different platelet membrane immunoprecipitates (Figure 1). One has been identified as fibrinogen, by crossed immunoelectrophoresis of fibrinogen alone, as well as fibrinogen mixed with the solubilized platelet membrane. Another has been identified as albumin, by similar methods. A third antigen has been identified as a 'Glanzmann's antigen(s)' since it is missing on platelets from a patient with Glanzmann's thrombasthenia. A fourth antigen is sensitive to neuraminidase and chymotrypsin treatment, and is probably glycopeptide 1.

The rabbit anti-human platelet membrane antibody can be completely adsorbed with 1×10^9 platelets (Figure 2a-k). Note the increase in the height of the immunoprecipitates with increasing adsorption of antibody following exposure to increasing numbers of platelets. The antibody cannot be adsorbed with a comparable volume of red blood cells, granulocytes or lymphocytes, demonstrating platelet specificity. We have identified at least 4 of these 19 antigens. Fibrinogen has been identified by electrophoresis of fibrinogen alone, membrane constituents alone, and fibrinogen plus membrane constituents (Figure 3a-c). Note the increased height of the fibrinogen peak. Similar results were obtained with albumin (Figure 4a-c). A third antigen has been identified as a 'Glanzmann's antigen(s)' since it appears to be missing on platelets from a patient with this disease (Figure 5a-c). Note the retention of the 1F fibrinogen peak, suggesting its relative absence on the patient's platelets. A fourth antigen is sensitive to neuraminidase and chymotrypsin treatment, and is probably glycopeptide 1 (Figure 6a,b). Note shift of 10 toward the anode with development of the right side of its peak; disappearance of 13 and 18; and apparent increase in height of 10 and 1F. Figure 7a,b demonstrates the effect of concanavalin A ligand. Those immunoprecipitates reacting with the ligand are pulled down into the spacer containing the ligand. Antigens 10, 13, 18, and 1F have been pulled down into the con A lectin, whereas 2CS, 3, 5 (barely visible) and 16 are not affected.

2. Requirement of Microtubules for Platelet Aggregation and Secretion: Association of Phosphodiesterase Activity with Platelet Tubulin.

In our previous Army Contract Report (19) we presented data on the effect of colchicine, D_2O , and cyclic AMP on platelet aggregation and protein phosphorylation: Platelets were incubated in the presence and absence of colchicine ($10^{-8}M$), D_2O (60-100%), $10\mu M$ dibutyl cyclic AMP (db cAMP), $1\mu M$ PGE₁ or $10mM$ NaF; prior to platelet aggregation induced by Ca ionophore A23817 ($1\mu g/ml$). Protein phosphorylation was measured by incubation with $1mC$ $^{32}PO_4/ml$. Cells were washed, sonicated, and the reduced cell sap applied to 10% SDS PAGE; gels were monitored for radioactivity and m.w. Colchicine completely inhibited the 2° wave of platelet aggregation (release) as well as phosphorylation of proteins 10-100,000 m.w. 3H -colchicine ($10^{-8}M$) was shown to specifically bind to tubulin by sephacryl gel filtration of cell sap (single peak, 110,000 m.w.) and 10% PAGE of isolated peak which coelectrophoresed with purified brain tubulin. D_2O completely overcame the colchicine inhibition of aggregation and

phosphorylation. Db cAMP also completely inhibited platelet aggregation; and db cAMP as well as PGE₁ and NaF markedly inhibited protein phosphorylation; however, D₂O did not reverse cAMP-inhibition of phosphorylation. These data indicate that unperturbed microtubules are necessary for protein phosphorylation and the 2° wave of platelet aggregation (release).

These data have been extended: colchicine and cyclic AMP both have now been shown to stimulate dephosphorylation of phosphorylated cell sap. Colchicine was shown to operate via elevation of cyclic AMP levels. Both colchicine and cyclic AMP have also been shown to inhibit the platelet release reaction. O₂O overcomes the inhibitory effect of colchicine.

Effect of Cyclic AMP on Phosphorylated Platelet Cell Sap. Because inhibitory agents were added 15 minutes prior to the addition of ³²P_{O₄} to the incubation medium and were therefore inhibiting incorporation of ³²P_{O₄} into platelet cell sap, we elected to determine what effect elevated cyclic AMP levels would have on platelet proteins which had already been phosphorylated. Accordingly, washed platelets were preloaded with ³²P_{O₄} for 30 minutes and then exposed to agents which would elevate intracellular cyclic AMP levels. These agents served to dephosphorylate the incorporated ³²P_{O₄} of platelet cell sap. Such was the case with 10 mM NaF, Figure 8a (representative of 3 experiments); 0.1 mM dibutyl cyclic AMP, Figure 8b; or 10 μM prostaglandin E₁, Figure 8c (representative of 4 experiments).

Effect of Colchicine and 2',5'-dideoxyadenosine (DDA) on Phosphorylated Platelet Cell Sap. Because colchicine appeared to have the same effect as cyclic AMP with respect to inhibition of platelet aggregation and inhibition of incorporation of ³²P_{O₄} into platelet cell sap, experiments were designed to determine whether colchicine was acting via cyclic AMP. Washed platelets were preloaded with ³²P_{O₄} for 30 minutes and then exposed to colchicine with or without 0.1 mM 2',5'-dideoxyadenosine (DDA), a potent inhibitor of adenylate cyclase (19). Figure 9a demonstrates that 10⁻⁷M colchicine also served to dephosphorylate the incorporated ³²P_{O₄} of platelet cell sap proteins (representative of 9 experiments), like cyclic AMP (see Figure 8). DDA had no effect by itself on phosphorylated platelet cell sap (Figure 9b) but completely prevented the dephosphorylation effect of colchicine (Figure 9c, representative of 5 experiments). Similar effects of 0.1mM DDA were obtained when platelets were incubated with 10 mM NaF (data not shown).

Effect of Colchicine on Intracellular Cyclic AMP Levels. The next experiment was designed to determine directly whether 10⁻⁷M colchicine had any effect on platelet intracellular cyclic AMP levels. Washed platelets (7.5 volumes %) were incubated at 37°C with and without 10⁻⁷M colchicine and O₂O for 15 minutes in the presence of 5 mM aminophylline and then treated with 6% TCA to extract cyclic AMP which was assayed by a competitive protein binding technique (20). Cyclic AMP levels in the absence of colchicine, 640 picomoles/ml platelet extract, increased to 875 picomoles/ml (2 experiments). D₂O (100%) prevented the rise in cyclic AMP levels which were 515 picomoles/ml in the absence of colchicine and 545 picomoles/ml in its presence (2 experiments).

Effect of D₂O and Colchicine on Platelet Aggregation and Release of Serotonin Induced by Ca⁺⁺ Ionophore A23187. D₂O, an agent known to stabilize microtubules in other tissues, shortened the Ca⁺⁺ ionophore-induced lag period by 43% and increased the velocity of platelet aggregation by 52% ± 14% (SEM) at a D₂O concentration of 60% (average of 7 experiments). The combination of 40% O₂O and colchicine, or D₂O and vinblastine resulted in partial correction of the alkaloid-induced inhibition of platelet aggregation velocity and prolongation of the lag period. For example,

at 40% D₂O, colchicine inhibited the H₂O control by 33%, whereas colchicine and D₂O inhibited the D₂O control by 18%; vinblastine inhibited the H₂O control by 45% whereas vinblastine and D₂O inhibited the D₂O control by 23%.

Table 1 demonstrates the effect of colchicine and D₂O on the platelet release reaction. Thus, ³C-serotonin release was inhibited by colchicine at 0.2-500 μM, following aggregation by Ca⁺⁺ ionophore at 5-10 μM. D₂O (44%) partially to completely prevented the colchicine-induced inhibition of ³C-serotonin release.

These data therefore indicate that unperturbed microtubules are necessary for protein phosphorylation, the secondary wave of platelet aggregation and the platelet release reaction. The data strongly suggest that intact microtubules serve to inhibit adenylate cyclase activity.

Evidence for the Association of cAMP Phosphodiesterase Activity with Platelet Microtubules. Our studies with intact platelets strongly suggest inhibition of adenylate cyclase activity by unperturbed platelet microtubules, since colchicine binds to platelet microtubules; colchicine operates like cyclic AMP; colchicine raises intracellular cyclic AMP levels; the effects of colchicine on platelet aggregation, secretion and phosphorylation can be reversed with D₂O, a stabilizer of microtubules; and colchicine's effect on phosphorylation can be reversed by 2',5'-dideoxyadenosine, an inhibitor of adenylate cyclase activity. We therefore propose that platelet tubulin may act as a phosphodiesterase or be closely associated with a protein with phosphodiesterase activity.

Preliminary studies suggest that this may be the case. We have partially purified platelet tubulin by modifications of the methods of Shelanski (21) and Steiner (22). This preparation contains phosphodiesterase activity.

We have assayed cAMP phosphodiesterase by measuring the release of Pi employing the phosphomolybdate reaction. A 350-fold purification preparation requires Mg⁺⁺ and gives a K_m of 77 μM for cyclic AMP. It does not react with 2 mM cyclic GMP and is strongly inhibited by 5 μM NaF (50% inhibition at 0.1 mM cAMP); as well as heavy metals in the mM range: Zn⁺⁺>Cu⁺⁺>Fe⁺⁺>Mn⁺⁺. This cAMP phosphodiesterase appears to be unique in that it is not inhibited by 10 mM caffeine, theobromine, theophylline, methyl isobutyl xanthine, or xanthine, whereas it is inhibited (50% inhibition) by 35 μM hypoxanthine, 150 μM papavarine and 250 μM aminophylline.

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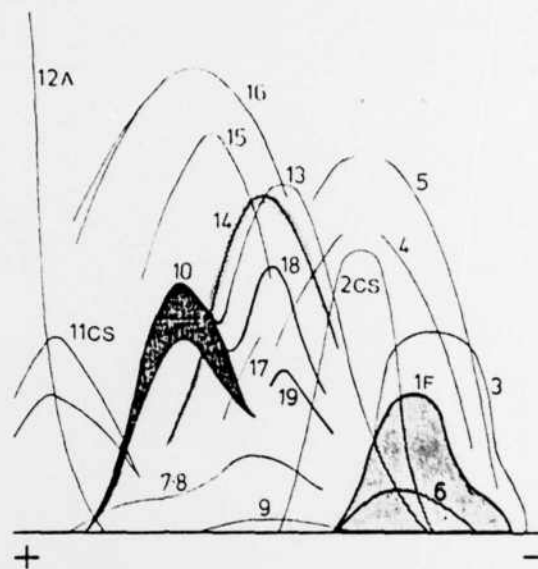


Figure 1a. Crossed immunoelectrophoresis of a human platelet membrane preparation. Platelet membranes were prepared by the methods of Barber and Jamieson (16) or Nurden and Caen (5), solubilized in 1% triton and (5ugm) electrophoresed on a 1% agarose slide in tris-barbital buffer, pH 8.6 for 3-4 hours at 150 volts. The slide was then rotated 90° and the platelet membrane antigens electrophoresed into freshly poured agar containing rabbit anti-human platelet membrane antibody (1:16 dilution of Ab) for 16 hr at 55 volts. The height of the peak is proportional to the Ag/Ab ratio. a) A representative diagram of all the peaks observed. Nineteen different antigens (immuno-precipitate peaks) have been observed. Peak 1F is fibrinogen; 12A is albumin; 13 and 18 are missing in a patient with Glanzmann's thrombasthenia; 10 is the major antigen which is rich in sialic acid, ?GP1; and 2CS and 11CS are cell sap antigens.



Figure 1b. CIE of a membrane preparation, employing the first batch of rabbit anti-human platelet antibody (Rab 1).

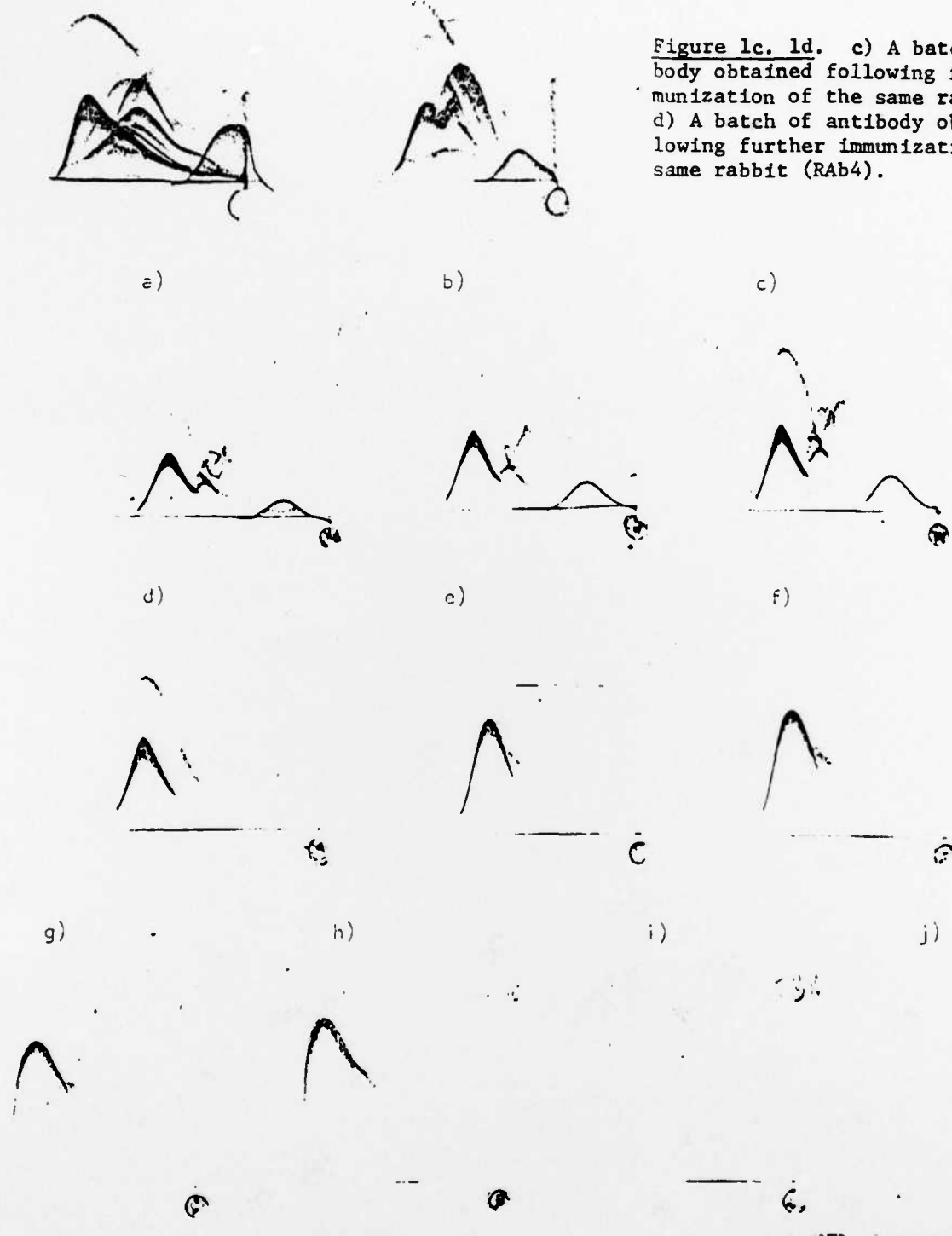


Figure 1c. 1d. c) A batch of anti-body obtained following further immunization of the same rabbit (Rab2). d) A batch of antibody obtained following further immunization of the same rabbit (Rab4).

Figure 2. Crossed immunoelectrophoresis of a human platelet membrane preparation following adsorption of rabbit anti-platelet membrane antibody with increasing concentration of washed human platelets. a) 0 platelets b) 6.7×10^7 platelets c) 1.25×10^8 platelets d) 2.5×10^8 platelets e) 5.0×10^8 platelets f) 6.0×10^8 platelets g) 7.0×10^8 platelets h) 8.0×10^8 platelets i) 1.0×10^9 platelets j) 1.2×10^9 platelets.

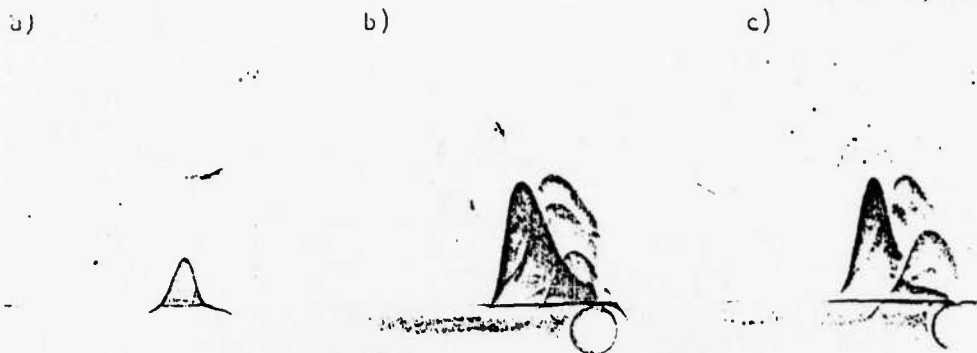


Figure 3. Crossed immunoelectrophoresis of a) human fibrinogen (0.12 μ gm); b) platelet membrane preparation; c) fibrinogen plus platelet membrane preparation. This CIE was run for a shorter time interval in the first dimension.

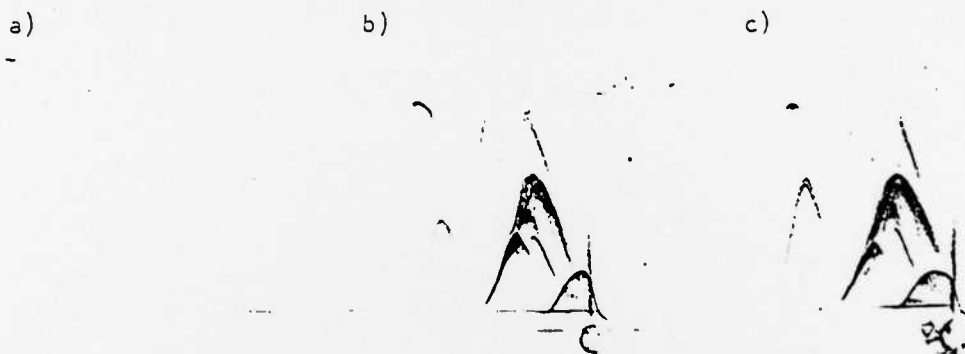


Figure 4. Crossed immunoelectrophoresis of a) human albumin (0.1 μ gm); b) platelet membrane preparation; c) albumin plus platelet membrane preparation.

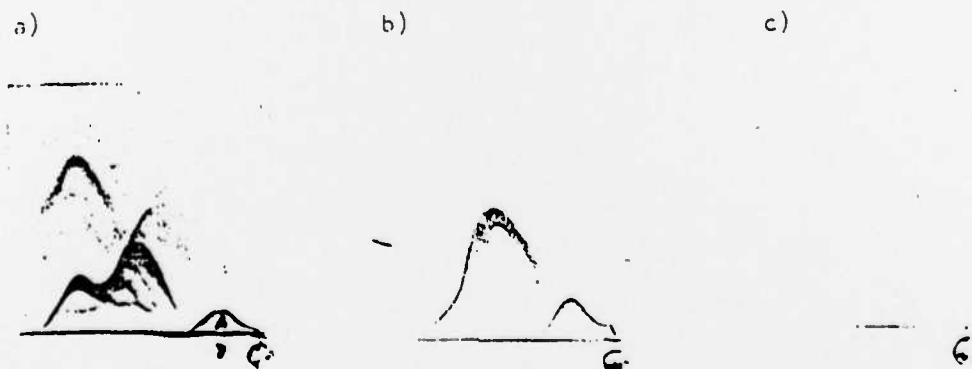


Figure 5. Crossed immunoelectrophoresis of a human platelet membrane with rabbit anti-platelet membrane antibody adsorbed with washed platelets from a patient with Glanzmann's thrombasthenia. a) unadsorbed antibody; b) antibody adsorbed with 1×10^9 platelets from a patient with Glanzmann's thrombasthenia; c) antibody adsorbed with 1×10^9 platelets from a normal subject.

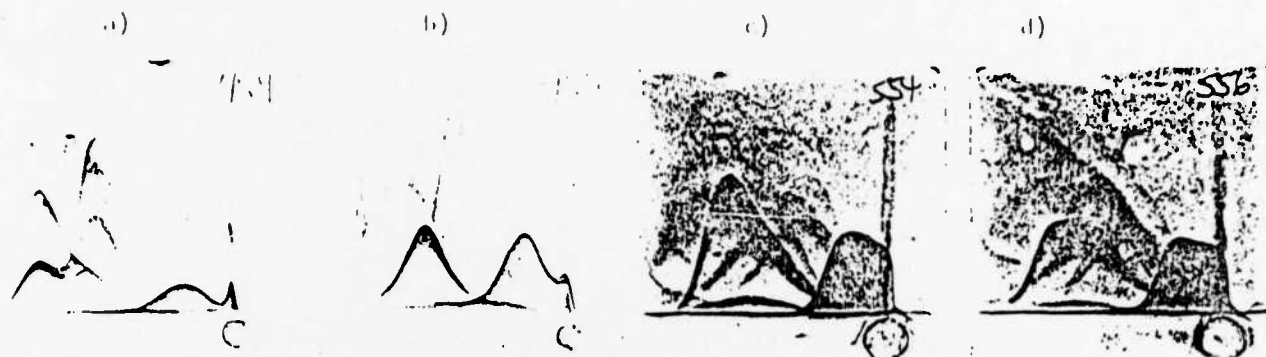


Figure 6. Crossed immunoelectrophoresis of a human platelet membrane preparation before and after treatment of the original washed platelets with neuraminidase. a) Washed platelets (8×10^8) treated with buffer: b) Washed platelets treated with neuraminidase, 2 units/ml. for one hour at 37°C . CIE of a human platelet membrane preparation before and after treatment of original washed platelets with α -chymotrypsin: c) 8×10^9 washed platelets treated with buffer: d) Washed platelets treated with 1mg/ml chymotrypsin for 20 min at 22°C . (Hazy peaks in background are artifacts due to fast running proteins in first dimension which enter buffer and cross-react with antibody in second dimension).

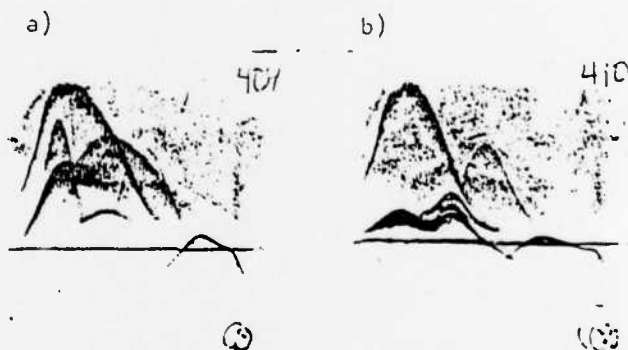


Figure 7. Crossed immunoelectrophoresis of a human platelet membrane preparation in the presence of a spacer gel (inserted with the second dimension a) containing agarose alone: b) containing concanavalin A (250 $\mu\text{g}/\text{ml}$) in agarose. Note antigens pulled down into the lectin. (Similar artifacts noted, as in Figure 6).

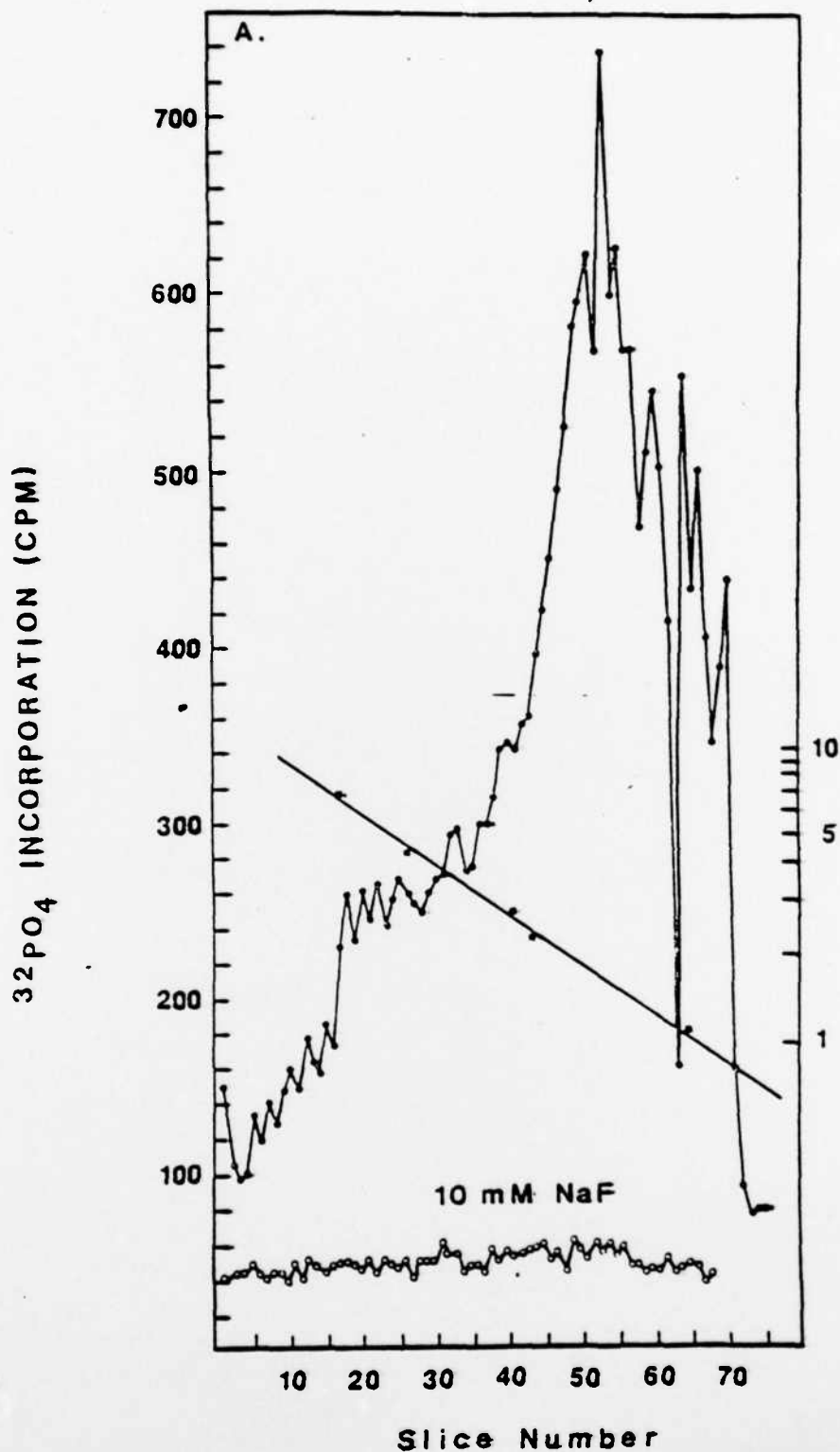


Figure 8A. Effect of NaF, dibutyl cyclic AMP, and prostaglandin E_1 on platelets preloaded with $^{32}\text{PO}_4$. Washed platelets were preincubated with 1 mc/ml $^{32}\text{PO}_4$ for 30 minutes and then washed twice in tris-buffered saline, pH 7.4 (see Methods). The platelets were then resuspended in buffer containing either buffer (●—●) or 10 mM NaF (o—o), and incubated for 30 min (all 4 incubations were performed with the same batch of platelets). The cell sap was prepared and electrophoresed on a 10% SDS polyacrylamide gel.

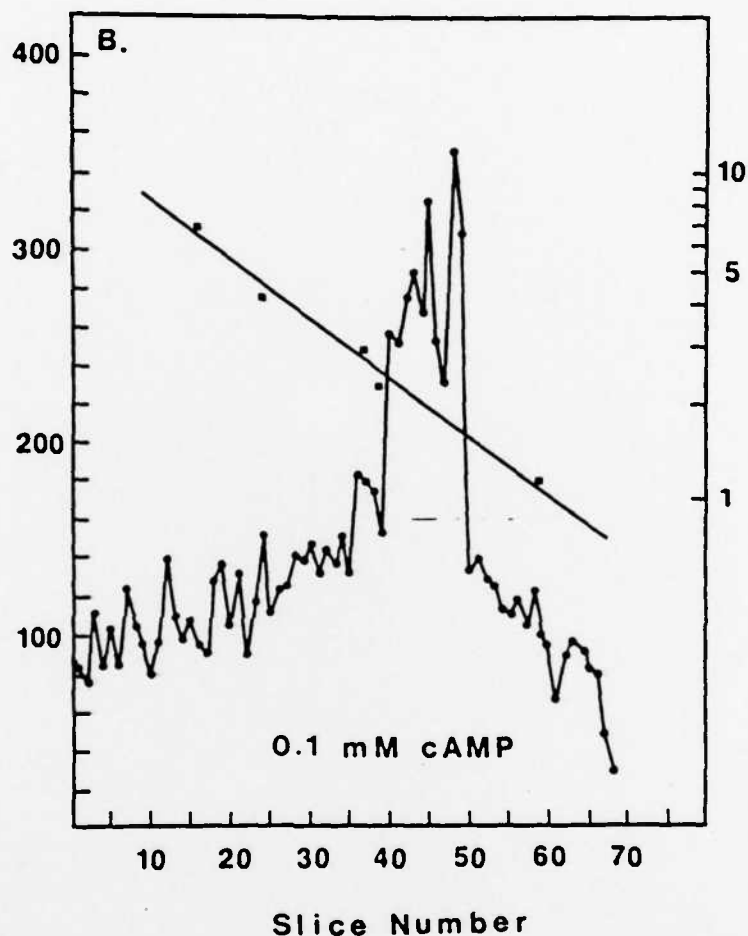


Figure 88. Effect of NaF, dibutyl cyclic AMP, and prostaglandin E_1 on platelets preloaded with $^{32}PO_4$. Washed platelets were preincubated with 1 mc/ml $^{32}PO_4$ for 30 minutes and then washed twice in tris-buffered saline, pH 7.4 (see Methods). The platelets were then resuspended in buffer containing 0.1 mM dibutyl cyclic AMP (●—●), and incubated for 30 min (all 4 incubations were performed with the same batch of platelets). The cell sap was prepared and electrophoresed on a 10% SDS polyacrylamide gel.

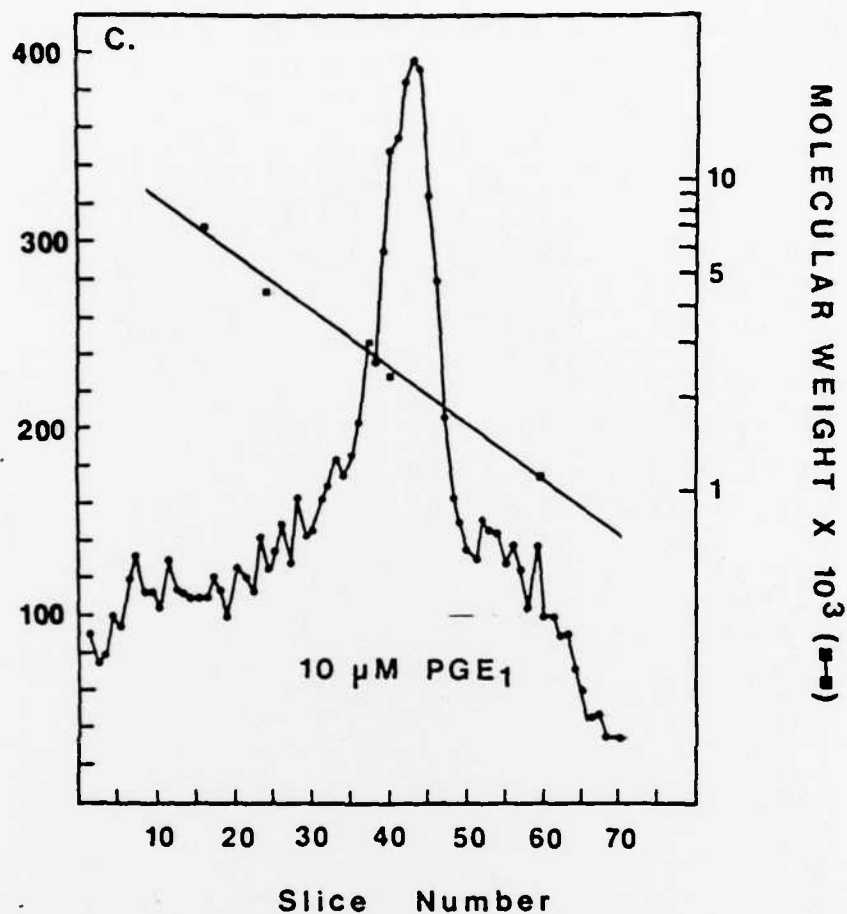


Figure 8C. Effect of NaF, dibutyl cyclic AMP, and prostaglandin E₁ on platelets preloaded with ³²P₀₄. Washed platelets were preincubated with 1 mc/ml ³²P₀₄ for 30 minutes and then washed twice in tris-buffered saline, pH 7.4 (see Methods). The platelets were then resuspended in buffer containing 10 μm prostaglandin E₁ (●-●) and incubated for 30 min (all 4 incubations were performed with the same batch of platelets). The cell sap was prepared and electrophoresed on a 10% SDS polyacrylamide gel.

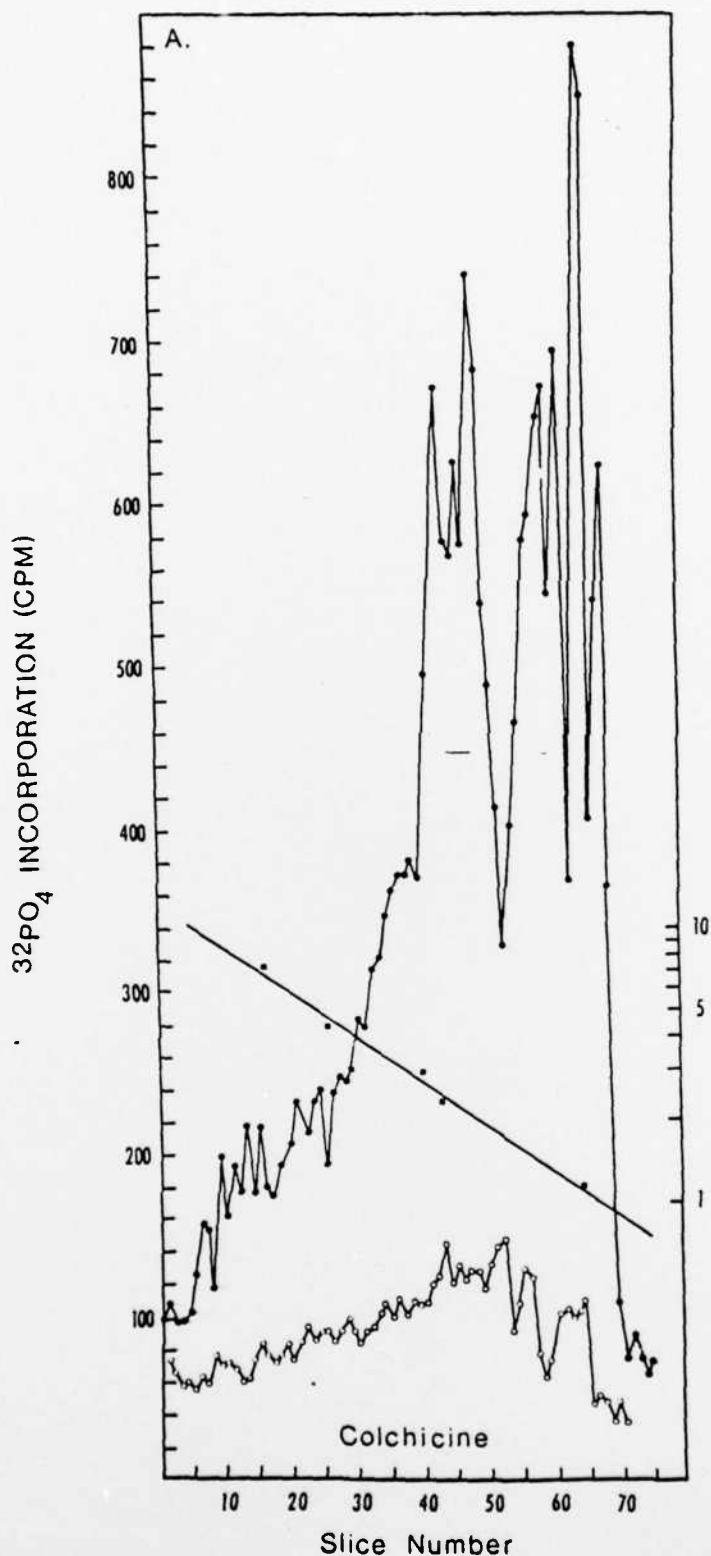


Figure 9A. Effect of colchicine and 2',5'-dideoxyadenosine (DDA) on platelets pre-loaded with $^{32}\text{P}_4$. Washed platelets were preincubated with 1 mc/ml $^{32}\text{P}_4$ for 30 min and then washed twice in tris-buffered saline, pH 7.4 (see Methods). The platelets were then resuspended in buffer containing either buffer (●—●) or colchicine at 10^{-7}M (○—○) and incubated for 30 min (all 4 incubations were performed with the same batch of platelets). The cell sap was prepared and electrophoresed on a 10% SDS polyacrylamide gel.

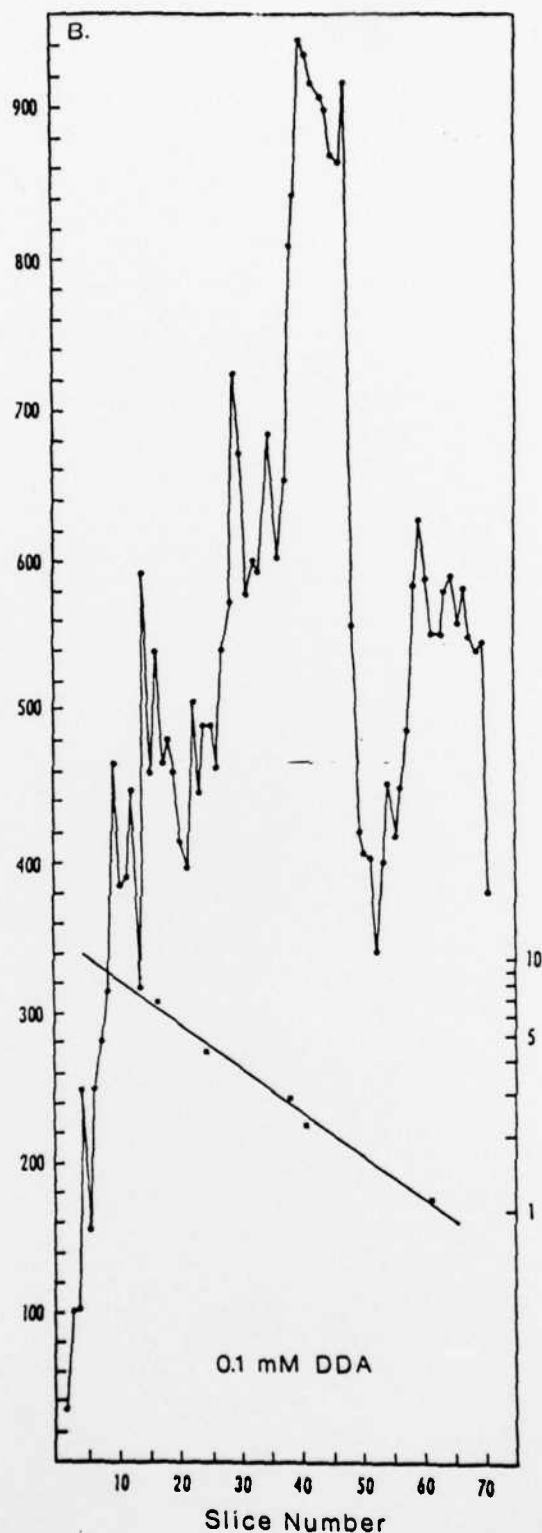


Figure 9B. Effect of colchicine and 2',5'-dideoxyadenosine (DDA) on platelets pre-loaded with $^{32}\text{P}\text{O}_4$. Washed platelets were preincubated with 1 mc/ml $^{32}\text{P}\text{O}_4$ for 30 min and then washed twice in tris-buffered saline, pH 7.4 (see Methods). The platelets were then resuspended in buffer containing 0.1 mM dideoxyadenosine and incubated for 30 min (all 4 incubations were performed with the same batch of platelets). The cell sap was prepared and electrophoresed on a 10% SDS polyacrylamide gel.

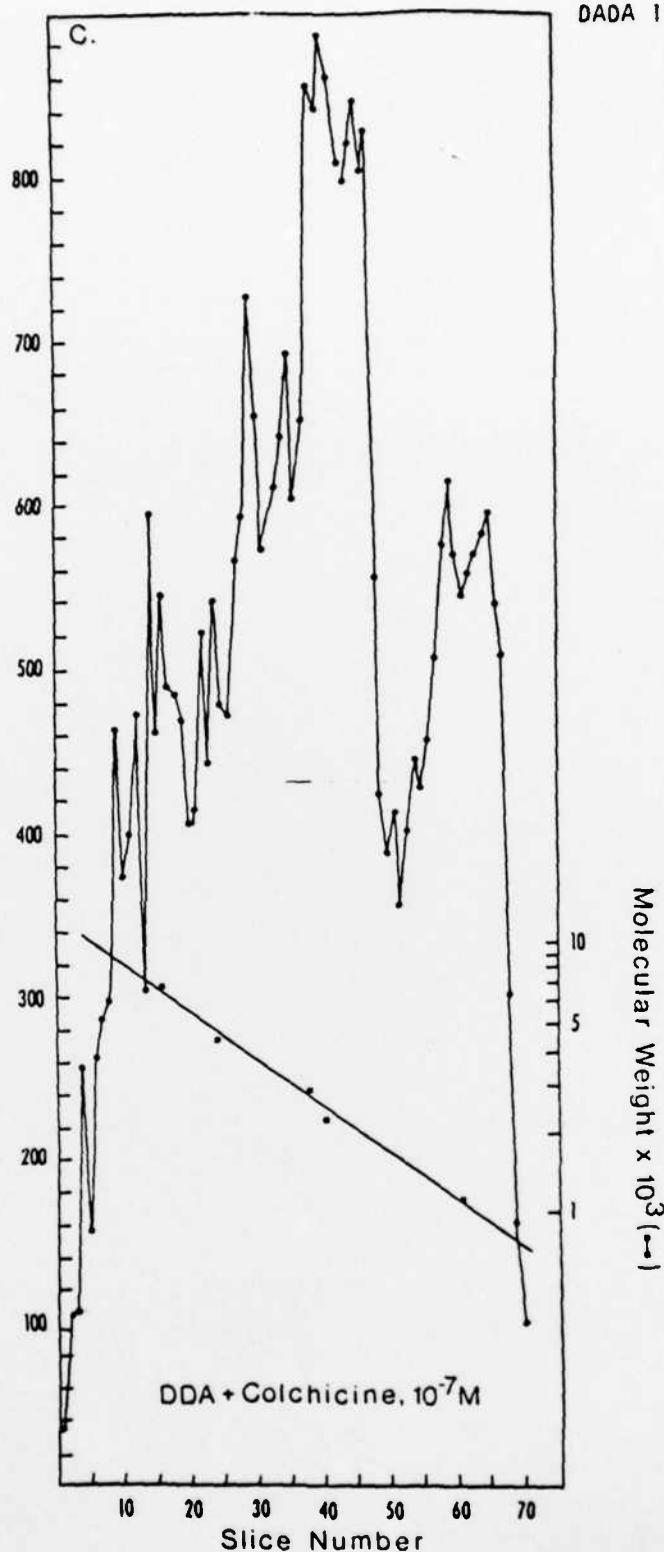


Figure 9C. Effect of colchicine and 2',5'-dideoxyadenosine (DDA) on platelets pre-loaded with $^{32}P_{O_4}$. Washed platelets were preincubated with 1 mc/ml $^{32}P_{O_4}$ for 30 min and then washed twice in tris-buffered saline, pH 7.4 (see Methods). The platelets were then resuspended in buffer containing colchicine at $10^{-7}M$ plus dideoxyadenosine, and incubated for 30 min (all 4 incubations were performed with the same batch of platelets). The cell sap was prepared and electrophoresed on a 10% SDS polyacrylamide gel.

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